SERUM OPSONIC ACTIVITY IN SPLENECTOMIZED HODGKIN'S DISEASE PATIENTS

by

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ABSTRACT

Overwhelming infection represents a significant hazard in patients following splenectomy or in functional asplenia due to a variety of disorders. Defects in the alternative complement pathway (ACP) activity have been suggested in several of these disorders. In the present study, nonspecific opsonic activity in Hodgkin's disease patients was investigated at various intervals postsplenectomy. A few patients were evaluated prior to surgery. This activity was measured indirectly by PMN chemiluminescence (CL) produced as the leukocytes phagocytize zymosan particles opsonized in chelated sera from patients and controls. ACP opsonic activity as measured by the CL produced was slightly depressed 10-31 days postoperatively but usually returned to normal levels one month after surgery in adults. In three adolescent patients, decreased opsonic activity persisted. No such depression was evident in nonchelated sera. Factor B and IgM levels did not correlate with the depressed activity. These data suggest that nonspecific opsonic activity of the ACP is transiently decreased in Hodgkin's disease patients following splenectomy; and, component(s) other than Factor B and IgM may be responsible for this abnormality. Additional investigations are indicated, however, to confirm these trends and correlate them with the propensity to develop overwhelming postsplenectomy infections.

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CHAPTER 1

INTRODUCTION

It has been shown repeatedly that splenectomized individuals have an increased incidence of overwhelming infections (4, 8, 16, 18, 20). Sickle cell anemia patients also have increased susceptibility to sepsis (11, 12, 21). Many theories have attempted to describe the abnormality which is responsible for such patients' susceptibility to organisms such as Streptococcus pneumoniae, Escherichia coli, and Hemophilus influenzae (12, 16, 18). As the spleen is considered to be the largest reticuloendothelial organ in the body, it is readily apparent that the loss or dysfunction of this organ should have some effect on the body's ability to clear bacteria from the blood stream. Many theories concerning overwhelming infection after the removal or dysfunction of the spleen have focused on aspects of the processes of bacterial clearance.

An important aspect of the body's defense mechanism results from phagocytosis and intracellular killing by the cells of the reticuloendothelial systems. Bacterial clearance by the phagocytes has been recognized to occur in four phases which include chemotaxis (the migration of the phagocyte toward the bacteria resulting from the attraction by chemotactically active substances), opsonization (the coating of the bacteria with substances which promote ingestion by the phagocyte), actual ingestion (the bacteria is taken inside the

phagocyte and stored in a vacuole), and degranulation (the lysosomal granules of the phagocyte release their contents into the vacuole which aids in killing of the bacteria) (19). The second phase opsonization, has been theorized to have several mechanisms depending upon the type of particle as well as the immune status of the host (19). Specific anticapsular antibody may act alone or in conjunction with the classical complement pathway to opsonize particles. In the absence of specific antibody, the alternative complement pathway (ACP) may act as a nonspecific opsonin (14, 19).

Splenectomized patients are often infected with organisms such as S. pneumoniae to which they may not possess high levels of specific antibody (12, 16, 18). When such antibody is not present, precise functioning of the alternative complement pathway (ACP) is imperative (12). Several investigators have reported defects in the ACP in splenectomized patients which may contribute to the increased incidence of overwhelming infection (11, 21). These abnormalities have been attributed to decreased levels of functioning Factor B (17), IgM (15), C3 (11), and a special protein, Tuftsin (5). Others using different methods have found no difference in opsonizing capacities via the ACP between functionally splenectomized patients and normal children (22). These studies point out the problems associated with current assays for opsonic activity.

Hill and workers (10) have shown that measurement of PMN chemiluminescence (CL) during phagocytosis of opsonized zymosan particles can be used to indirectly quantitate ACP opsonic activity. In this procedure, zymosan particles are opsonized in serum treated

with Mg^{++}- ethyleneglycol tetraactic acid (EGTA) to remove calcium thus blocking the classic complement pathway (6, 7, 10, 17). The opsonized zymosan particles are then mixed with a suspension of normal PMNs. As the PMNs phagocytize the particles, there is an increase in leukocyte hexose monophosphate shunt activity producing excited molecular oxygen and carbonyl groups. As these excited molecular forms relax to the ground state, photons are emitted which can be detected and quantitated using a liquid scintillation counter (1 2).

In the present study, we have used this method to assess nonspecific opsonic activity in serum from 21 splenectomized Hodgkin's disease patients. A few patients were evaluated before surgery in order to eliminate the possibility of abnormal opsonic activity due to Hodgkin's disease. Additional patients were drawn at various intervals after surgery to monitor any changes in opsonic activity with time. Factor B and IgM levels have also been measured.

CHAPTER 2

METHODS AND MATERIALS

Chemiluminescence was measured immediately following the addition of a suspension of normal PMNs to zymosan particles which have been opsonized in fresh or fresh frozen, chelated or unchelated serum from patients and controls. Prior to opsonization, the zymosan was washed and brought to proper concentration, and was refrigerated until the addition of serum and chelators.

Factor B and IgM levels were tested on serum samples drawn simultaneously for the chemiluminescence procedure. These levels were measured using immunodiffusion techniques (Behring Diagnostics, Sommerville, N.J.) of some patients and controls. No further discussion of immunodiffusion techniques will be made since no modification of this procedure was made and the focus of this study was chemiluminescence.

Serum

Venous blood obtained from Hodgkin's disease patients and age-matched controls was allowed to clot over a 30 minute period. The serum was separated and stored immediately at -70°C until assay.

Leukocyte Suspensions

Heparinized (10 U/ml) whole venous blood was obtained from healthy adult donors. Leukocyte rich plasma was collected using

dextran sedimentation. The leukocyte rich plasma was centrifuged at 1,000 rpm for 10 minutes, and the supernatant was removed. The remaining cell button was washed twice in sterile phosphate buffered saline (PBS), (pH 7.4) (4,500 ml distilled water, 5.2g Na_2PO_4, 0.9g KCl, 0.9 g KH_2PO_4, and 36g NaCl). The cell button was resuspended in PBS, counted using a hemacytometer, and adjusted to 1 x 10^7 PMN/ml. This suspension was prepared just prior to addition of zymosan.

Zymosan Preparation

Zymosan particles (Schwartz-Mann, Orangeburg, N.Y.) were washed once in sterile PBS and adjusted to a concentration of 10mg/ml in PBS. One-half ml aliquots were dispensed in sterile 12 x 75mm plastic tubes (Falcon, Oxnard, Calif.) and centrifuged at 2,000 rpm for 10 minutes. The PBS was removed, and the pellets were stored at 4°C for no longer than one week.

Opsonization of Zymosan

The zymosan pellet was reconstituted with 0.5ml of freshly thawed unchelated serum or 0.4ml freshly thawed serum to which 50ul of 100mM EGTA (Sigma, St. Louis, Mo.) and 50ul of 10mM $MgCl_2$ were subsequently added. The tubes were rotated 1 hour at 37°C in a rotorack (Fisher Scientific, Santa Clara, Calif.). After rotation, zymosan was centrifuged at 2,000 rpm for 10 minutes. The serum was removed and replaced with 0.5ml PBS.

Quantitation of Chemiluminescence

Two tenths ml of opsonized zymosan particles in PBS (10mg/ml) were added to 0.5ml of the leukocyte suspension (1 x 10^7 PMN/ml)

and 2.8ml of PBS (total volume is 3.5ml) and placed scintillation vials wrapped in aluminum foil and stored in darkness for at least 18 hours prior to use. Vials were immediately capped, mixed, and placed in a Beckman LS-100c scintillation counter (Fullerton, Calif.), out of phase, with one photomultiplier tube disconnected. Each reaction was counted starting at 1 minute for 1 minute at 10 minute intervals for a total of 60 to 90 minutes. Duplicate samples were counted for each patient and control serum. Vials of only zymosan particles opsonized by control serum in PBS were counted along with vials containing only leukocyte suspension in PBS. CL was expressed as mean counts per minute of duplicate vials.

CHAPTER 3

RESULTS

Influence of Mg^{++}-EGTA on Chemiluminescence

These findings will be divided into 3 parts each discussing an aspect of this investigation: Influence of Mg^{++}-EGTA on chemiluminescence, relationship of opsonic activity to postsplenectomy interval, and correlation of opsonic activity with Factor B and IgM levels.

The effect of Mg^{++}-EGTA serum chelation on CL quantitation is illustrated in Figure 1. Actual accounts obtained from a representative patient and control are plotted against time. Lower peak CL values after treatment of Mg^{++}-EGTA are noted in both patient and control. The reason for this finding is unclear, but may indicate some contribution of the classic pathway to the opsonization of zymosan.

Figure 1 also illustrates the baseline counts of the PMN suspension and the opsonized zymosan particle control. No peak in CL is evident in these suspensions when they are counted separately. In contrast, when zymosan and PMNs are mixed, a marked peak in CL occurs. The leukocyte suspension resulted in an average background count of 4.7 ± 0.3 (SEM) $\times 10^3$ cpm. The zymosan particles opsonized by Mg^{++}-EGTA treated sera gave an average background count of $6.5 \pm$

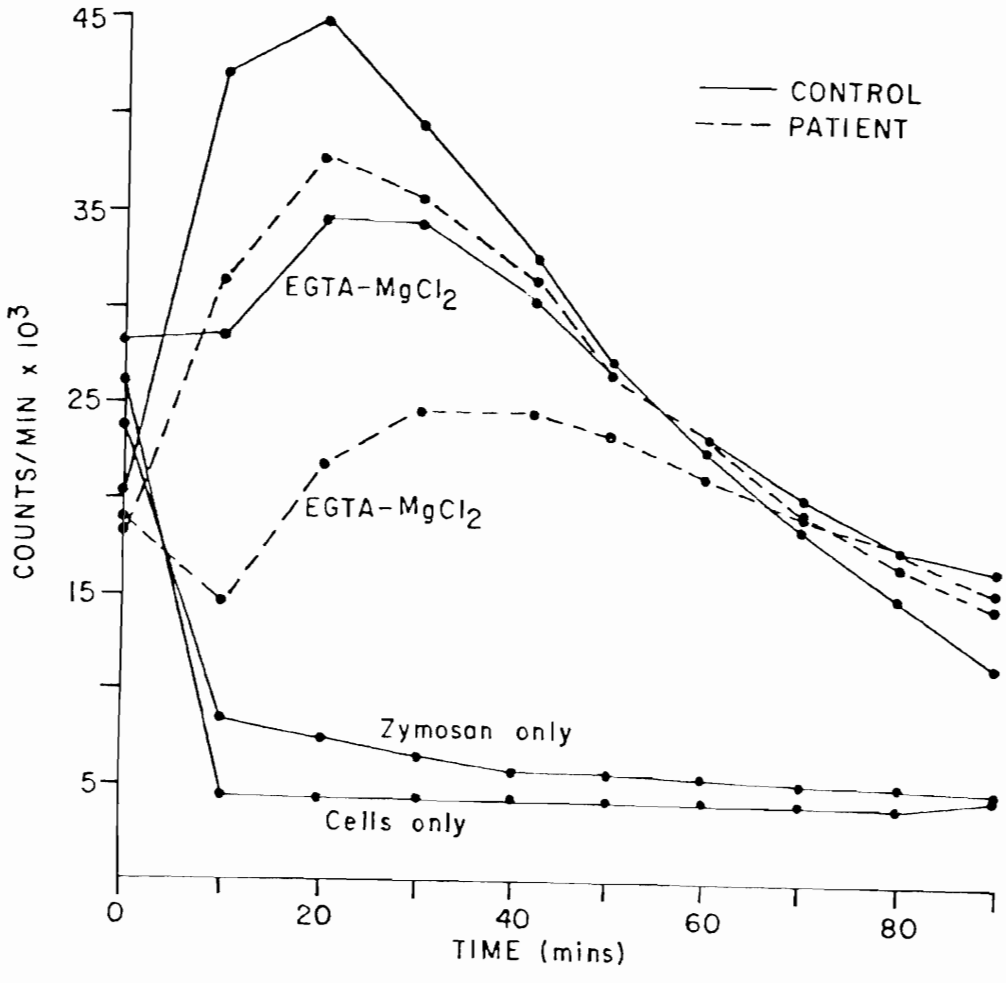

Figure 1. Effect of Mg++-EGTA serum chelation on CL. CL counts are plotted against time. The solid lines indicate the control counts and the dotted lines indicate representative patient counts. The counts obtained in chelated samples are designated. The zymosan and cell background counts are shown in the lower portion of the figure.

0.3 (SEM) x 10^3 cpm.

Figure 2 shows the effect of Mg^{++}-EGTA treatment on the kinetics of the CL peak. The average peak in CL occurred at approximately 25 minutes when zymosan was opsonized in untreated patient and control sera. Mg^{++}-EGTA treatment markedly delayed the CL peak to approximately 55 minutes for both groups. Again, the reason for this apparent delay in kinetics is unclear.

Relationship of Opsonic Activity to Postsplenectomy Interval

No marked differences were noted between ACP opsonic activity of patient and control sera when all specimens were considered. When sera from patients categorized by postsplenectomy interval were considered, some differences were observed. In Figure 3, each patient's actual count was compared to the corresponding control and expressed as a percentage of control. The first group, presplenectomy, did not differ from controls, 108.8%$\pm$4.6 (SEM). The second group consisting of patients from 10 to 31 days postsplenectomy showed a lower average peak CL value with a mean of 80.3% $\pm$4.6 (SEM). The second group consisting of patients from 10 to 31 days postsplenectomy showed a lower average peak CL value with a mean of 30.3%$\pm$2.6 (SEM). The controls for this group actually produced a somewhat higher mean value when compared to the other group controls. The reason for this is not evident but should be considered in evaluation of the results although CL is subject to some variance in counts between assays. The third group of patients were studied 32 days to 1 year postsplenectomy and produced an average peak of

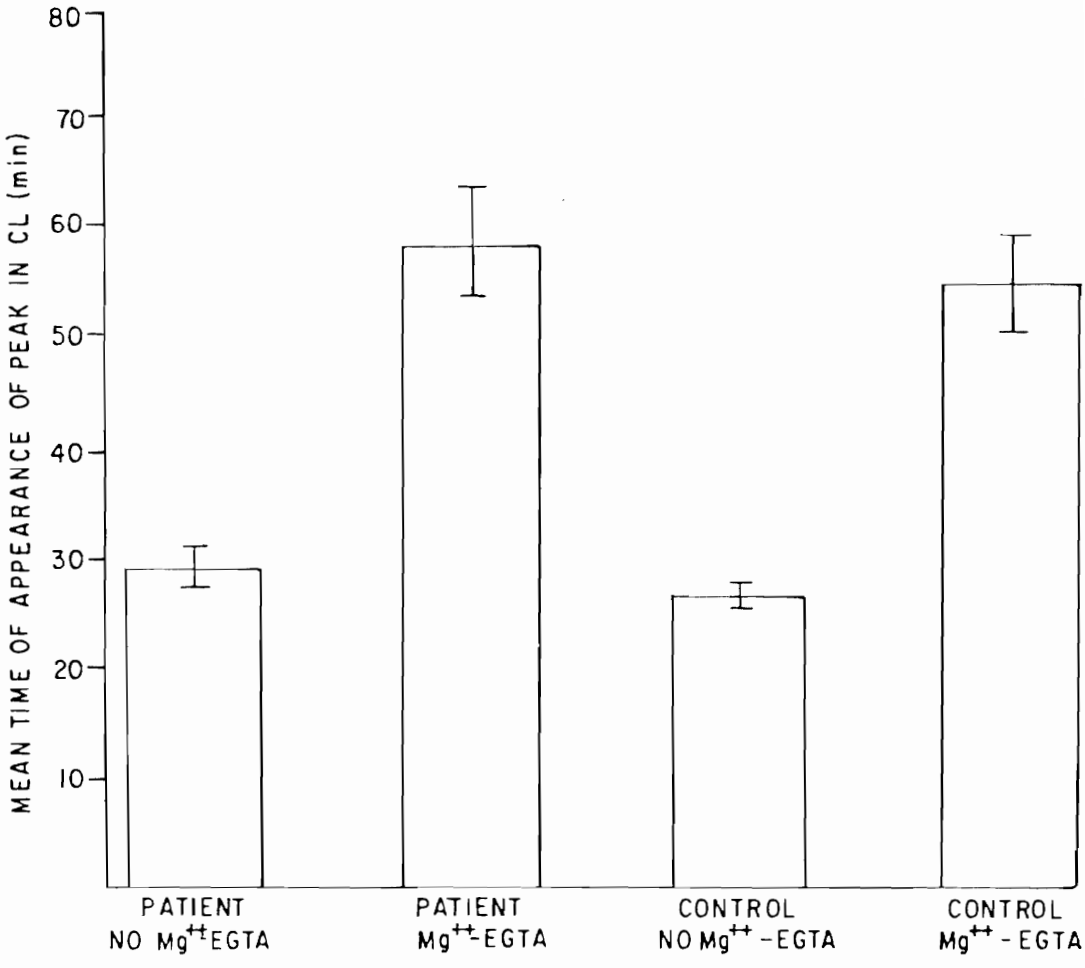

Figure 2. Effect of Mg++-EGTA serum chelation on the kinetics of peak CL for patients and controls. Mg++-EGTA treated groups have a delayed appearance of the peak.

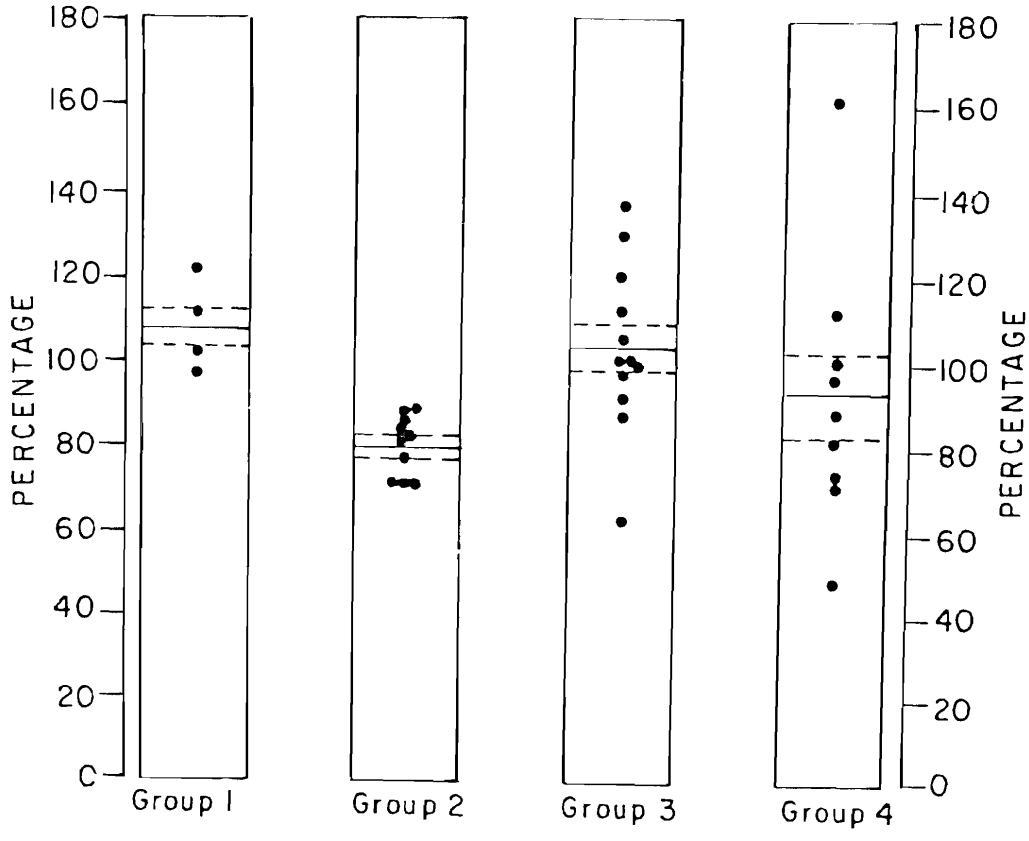

Figure 3. CL peak values categorized as to time before and after splenectomy. The CL values are expressed as percent of the corresponding control peak values. The solid lines indicate the mean peak values and the dotted lines represent the SEM.

104.3% ± 5.7 (SEM). A fourth group of patients from 1 to 6 years postsplenectomy were evaluated and produced a mean peak of 93% ± 10.8 (SEM).

No large differences were apparent, therefore, between adult patients and controls more than 1 month after splenectomy. There were, however, 3 adolescent patients who exhibited persistently low opsonic activity. A twelve year old female had low CL activity for over 2 months postsplenectomy (Table 1). A 15 year old male exhibited a CL value of 71% of the corresponding control, 2 years postsplenectomy; and, a 17 year old who had been splenectomized 5 years previously elicited a peak CL value of 49% of the control.

Table 1

CL Activity of Chelated Serum from a 12-year-old Hodgkin's Disease Patient Obtained at Various Intervals After Splenectomy. CL Values are Expressed as Percent of the Corresponding Control.

Days Postsplenectomy	9 days	31 days	63 days	121 days
Percentage of Control	96%	71.2%	64.4%	103.8%

Figure 4 depicts the kinetics of CL activity following mixture of zymosan, opsonized by Mg^{++}-EGTA treated sera, and the leukocyte suspension. The results of splenectomized Hodgkin's disease patients are plotted with time following splenectomy and shown as percentage of their corresponding control time of peak CL. Whenever a large drop in CL peak values was noted, there was also a

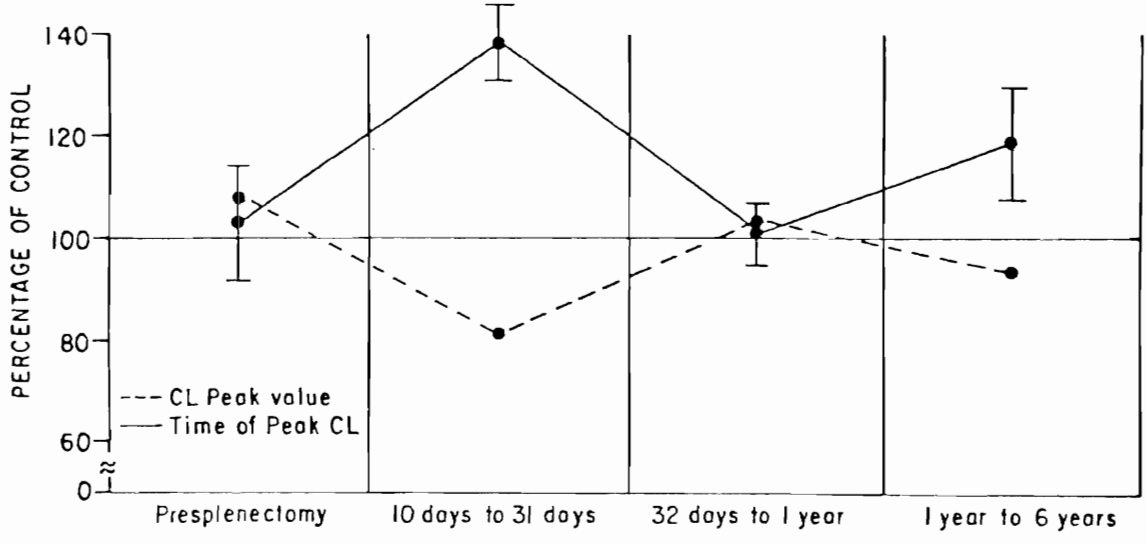

Figure 4. The time to the peak appearance of CL activity. The patients were grouped as to the time pre and post splenectomy. The patient values are expressed as percent of the corresponding control time. The mean values for each group are plotted, and the SEM are indicated for each group by the extending solid lines. The dotted lines represent the mean CL peak values expressed as percent of control peak values.

delay in the appearance of that peak. This suggests that the kinetics proceeded at a lower rate in the patients. At the time after splenectomy when the CL peak returned to values comparable to control, there was no delay in the time of peak CL activity.

Correlation of Opsonic Activity With Factor B and IgM Levels

Table 2 depicts the comparison of some CL values (expressed as percentages of control peak counts) with Factor B and IgM levels. No trends are evident in the comparison. Low CL values do not correspond low Factor B levels just as low IgM levels do not correspond with low CL values.

Table 2

The Correlation of CL Activity with Factor B and IgM Levels. CL Values are Expressed as Percent of the Corresponding Control Values. Normal Levels for Factor B are 12-30 mg/dl and Normal Levels for IgM are 60-299 mg/dl.

	CL % of Control Value	Factor B mg/dl	IgM mg/dl
	41.2	48.0	132
	71.0	81.6	20
	49.3	28.8	38
	71.3	23.0	102
	119	50.4	144
	121.9	21.6	26
	73.8	36.0	60
	88.4	78.4	108
	85	30.4	50
	130.4	25.2	66
	91.9	36.0	26
	163.7	102.4	50
Normal CONTROL 1		21.6	66
Normal CONTROL 2		19.6	168

CHAPTER 4

CONCLUSIONS

Discussion

It has been shown that the incidence of overwhelming sepsis increases from 0.1% in the normal population to 11.5% in splenectomized patients with reticuloendothelial diseases including Hodgkin's disease (16). Chilcote et al. has reported a 10% increase in the incidence of overwhelming postsplenectomy infections in a study of 200 Hodgkin's disease children and adolescents (4). Others have reported an increased incidence of overwhelming sepsis in disorders associated with functional asplenia such as sickle cell anemia (11, 12, 21). Johnston et al. (11) and Winklestein and Drachman (17) have proposed that sickle cell disease patients possess an inability to fully utilize the ACP during phagocytosis of S. pneumoniae which may explain, in part, the propensity to infection with this organism.

The present studies suggest that there may be a slight depression of nonspecific opsonic activity for 10 to 31 days after splenectomy in Hodgkin's disease patients (Figure 3). Since the control group for this category did not correlate with other control groups, the validity of this finding should be questioned at this time; however, the question may be resolved with further study and a greater number of assays. Nevertheless, some comment should be made concerning the trends that have become evident during this study.

Opsonic activity of unchelated patients' sera did not differ appreciably from control activity as illustrated by peak value (Figure 1) or CL kinetics (Figure 2). Chelated serum samples from the same assays did show some differences which suggest that the mechanisms involved in the opsonization of zymosan with and without chelation are not well defined (6).

Factor B levels obtained during this period of apparent depression of opsonic activity remained at control levels (Table 2). It has been previously shown by other investigators (10, 17) that low Factor B levels are associated with low ACP nonspecific opsonic activity in cord serum. However, in our population, no correlation was evident between Factor B levels and CL. IgM levels also remained at normal levels in most cases which disagrees with previous work (15). Therefore, if the trend continues, it appears that some component(s) in the nonspecific activity of the ACP other than Factor B may be deficient for a period of time following splenectomy in Hodgkin's disease patients.

Previous investigators have shown that the patient's age and time after splenectomy are both important factors in determining the susceptibility to overwhelming infection (20). The trends in our study suggest that the spleen may have an important role in the maintenance of some component(s) of the ACP and nonspecific opsonization (Figure 3 and Table 2).

During the period of study, none of the splenectomized Hodgkin's disease patients developed overwhelming infection. Although we have shown by an indirect measurement that a trend of abnormal

ACP nonspecific activity may exist in Hodgkin's disease patients following splenectomy, its significance in the observed susceptibility to infections remains to be determined.

Summary

This study indicates that splenectomy in Hodgkin's disease patients may cause depression in the nonspecific alternative complement pathway (ACP) opsonic activity for a period of time. The nonspecific ACP activity was measured indirectly by PMN chemiluminescence (CL) produced as the leukocytes phagocytize zymosan particles opsonized in chelated sera. A few patients drawn prior to surgery did not show the depression, and a few adolescent patients continued to have low CL values. Nonchelated sera did not result in such depressed activity. Factor B and IgM levels did not correlate with low CL values suggesting possible abnormalities in some other component(s) of the ACP. More study is indicated in order to confirm these trends. Since none of the splenectomized patients developed overwhelming infection during our study, additional investigations are necessary to more positively correlate these trends with the patients' propensity to overwhelming postsplenectomy infections.

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